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(54) Methods and means for inducing apoptosis by interference in RNA processing

(57) The invention relates to activation of apoptosis by means of interference with the function of snRNPs and hnRNP-like compounds.

Also the invention relates to anti-tumor therapies with compounds, which negatively interfere with sn-RNPs and hnRNP-like compounds leading to induction of apoptosis, resulting in the elimination of tumor cells.

Also the invention relates to therapies for diseases related to aberrant apoptosis induction, such as auto-immune diseases.

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be used as diagnosis of cancer-prone cells and treatment of cancer-prone cells.

[0012] Knowing that apoptin is quite safe in normal cells, but that as soon as a cell becomes transformed and/or immortalized (the terms may be used interchangeable herein) the present inventors designed novel menans and methoids for induction of apoptosis based on the identification of compounds involved in the apoptin-induced apoptotic cascade. These compounds are factors of an apoptosis pathway, which is specific for transformed cells. Therefore, these proteins are very important compounds in new treatments and diagnosis for diseases related with aberrancies in the apoptotic process, such as cancer, and (auto-)immune diseases. A group of proteins found to be associated with apoptin is the family of hnRNP-like proteins.

[0013] The invention provides an apoptin-associating hnRNP-like protein, which is needed for RNA processing. When apoptin associates with such proteins it interferes with normal RNA processing, thus leading to apoptosis.

[0014] The invention thus further provides a method for inducing apoptosis through interference with hnRNP-like proteins (interchangeably referred to as hnRNP or hnRNP-like proteins) or other parts of hnRNP's.

[0015] The invention provides an anti-tumor therapy based on the interference with hnRNP-like proteins or other parts of hnRNP's.

As an additional mechanism hnRNP can shuttle apoptin or apoptin-like compounds to the nucleus where these compounds can induce apoptosis.

The invention thus provides hnRNP as mediator of apoptin-induced apoptosis, which is tumor-specific.

[0016] The present inventors have also shown a colocalization of VP2 with snRNP's another compound also involved in RNA processing.

[0017] The invention provides a VP2-associating snRNP-like protein or component, which is needed for RNA processing.

[0018] The invention further provides a method for inducing apoptosis through interference with snRNP-like proteins or components (interchangeably referred to as snRNP or snRNP-like proteins).

[0019] The invention provides an anti-tumor therapy based on the interference with snRNP-like proteins.

[0020] The invention provides snRNP as mediator of VP2-induced apoptosis.

[0021] The invention further provides a method for inducing apoptosis through interference with hnRNP-like and snRNP-like proteins.

[0022] The invention provides an anti-tumor therapy based on the interference with either or both snRNP- and hnRNP-like proteins.

[0023] The invention provides hnRNP and snRNP as mediators of VP2-induced apoptosis.

[0024] More in detail the invention provides a recombinant and/or isolated nucleic acid molecule encoding a member of the family of hnRNP proteins involved in RNA processing comprising at least a functional part of the sequence of figure 1 or a sequence having at least 60, preferably 70, preferably 80, more preferably 90% homology with said sequence. In cells where a particular hnRNP is not used for RNA processing such hnRNP activity can be used to shuttle apoptotic agents such as apoptin to the nucleus. It is then preferred to have such activity in an expression vector. hnRNP (-like) activity is defined as any molecule directly or indirectly providing the same kind of activity as an hnRNP or an hnRNP-like protein.

[0025] Such a vector preferably also encodes apoptotic activity, preferably apoptin-like acctivity which is defined analogous to hnRNP-like activity.

In this definition functional equivalents and/or fragments of apoptin are also encompassed.

[0026] In the case where hnRNP's are involved in RNA processing these compounds can be inhibited by apoptin-like activity, but also by for instance antisense molecules for hnRNP components. The invention thus also provides a recombinant and/or isolated nucleic acid molecule encoding an antisense recombinant molecule which can hybridize with a recombinant acid molecule according to claim 1. Preferably again such a molecule is present in an expression vector.

[0027] Apoptosis is preferably induced in a gene therapy setting, so that it is preferred to deliver all vectors to cells making use of gene delivery vehicle. Gene delivery vehicle are known in the art and our capable of transporting nucleic acid molecules of interest to cells. They include recombinatn viruses (such as adenoviruses and retroviruses) as well as polymers and liposomes and the like.

It is preferred to also block the snRNP involvement in RNA processing. This can be done by VP2 (or VP2-like activity (same defintion as hnRNP-like activity)) or by a further antisense molecule hybridizing with a nucleic acid molecule encoding a snRNP component.

Both options are provided by the present invention. The invention thus provides an expression vector encoding an antisense molecule for a nucleic acid encoding a component of an snRNP, preferably together with an hnRNP antisense molecule.

[0028] The invention also provides a method for identifying apoptotic agents comprising the use of nucleic acid molecules encoding members of the hnRNP-like family and the snRNP-like family.

Apoptotic agents identified by such a route are also considered part of this invention. These agents will typically be hnRNP antagonists or snRNP antagonists of which apoptin and VP2 are the first examples.

The most preferred method of inducing apoptosis is using antagonists to both snRNP and hnRNP, but often singel anatagonists will suffice.

[0029] The invention will be explained in more detail in the following experimental part. This only serves for the purpose of illustration and should not be interpreted as a limitation of the scope of the invention.

coding apoptin-associating proteins were sequenced using dideoxy NTPs according to the Sanger method which was performed by Eurogentec, Nederland BV (Maastricht, The Netherlands). The used sequencing primer was a pACT-specific 17-mer comprising of the DNA-sequence 5'-TACCACTACAATGGATG-3'.

[0045] The sequences of the apoptin-associating proteins were compared with known gene sequences from the EMBL/Genbank.

Results and discussion

[0046] Apoptin induces specifically apoptosis in transformed cells, such as cell lines derived from human tumors. To identify the essential compounds in this cell-transformation-specific and/or tumor-specific apoptosis pathway, a yeast genetic screen was carried out.

[0047] We have used a human cDNA library, which is based on the plasmid vector pACT containing the complete cDNA copies made from Epstein-Barr virus-transformed human B cells (Durfee et al., 1993).

Construction of a bait plasmid expressing a fusion gene product of GAL4-DNA-binding domain and apoptin

[0048] To examine the existence apoptin-associating proteins by the human transformed/tumorigenic cDNA library, a so-called bait plasmid had to be constructed.

[0049] To that end, the complete apoptin-encoding region, flanked by about 40 basepairs downstream from the apoptin gene, was cloned in the multiple cloning site of plasmid pGBT9.

[0050] The final construct, called pGBT-VP3, was analysed by restriction-enzyme analysis and sequencing of the fusion area between apoptin and the GAL4-DNA-binding domain.

A gene(fragment) encoding an apoptin-associating protein is determined by transactivation of a GAL4-responsive promoter in yeast

[0051] The apoptin gene is fused to the GAL4-DNA-binding domain of plasmid pGBT-VP3, whereas all cDNAs derived from the transformed human B cells are fused to the GAL4-activation domain of plasmid pACT. If one of the cDNAs will bind to apoptin, the GAL4-DNA-binding domain be in the vicinity of the GAL4-activation domain resulting in the activation of the GAL4-responsive promoter, which regulates the reporter genes HIS3 and LacZ.

[0052] The yeast clones containing plasmid expressing apoptin and a plasmid expressing an apoptin-associating protein(fragment) can grow on a histidine-minus medium and will stain blue in a beta-galactosidase assay. Subsequently, the plasmid with the cDNA insert encoding the apoptin-associating protein can be isolated and characterized.

[0053] Before we could do so, however, we have determined that transformation of yeast cells with pGBT-VP3 plasmid only or in combination with an empty pACT vector, did not result in the activation of the GAL4-responsive promoter.

Identification of apoptin-associating proteins encoded by cDNAs derived from a human transformed B cell line

[0054] We have found yeast colonies, which upon transformation with pGBT-VP3 and pACT-CDNA were able to grow on a histidine-minus medium (also lacking leucine and tryptophan) and stained blue in a beta-galactosidase assay. These results indicate that these yeast colonies contain besides the bait plasmid pGBT-VP3 a pACT plasmid encoding for a potential apoptin-associating protein.

[0055] Plasmid DNA was isolated from these positive yeast colonies, which were transformed in bacteria. By means of an filter-hybridization assay using a pACT-specific labeled DNA-probe, the clones containing pACT plasmid could be determined. Subsequently, pACT DNA was isolated and digested with restriction enzyme XhoI, which is indicative for the presence of a cDNA insert. Finally, the pACT plasmids with a cDNA insert were sequenced.

Description of apoptin-associating proteins

[0056] The yeast genetic screen for apoptin-associating proteins resulted in the detection of a human homolog of the hnRNP-H. The determined DNA sequence is shown in Fig. IThe amino acid sequence of the cloned hnRNP-H homolog is shown in Fig.2. Most likely, the cloned cDNA insert represents a new member of the family of (human) hnRNPs.

Characteristics of hnRNP-H

[0057] The detected cDNA shows homology to part of hnRNP 1H, which is the abbreviation of heterogenous nuclear ribonucleoprotein H). hnRNPs bind to primary RNA transcripts (hnRNA or pre-mRNA), and are among the most abundant proteins in the nucleus. More than 20 hnRNPs have been discovered sofar, differing in size, localization, domains and nucleic acid binding specificity. Some hnRNPs were found to be confined to the nucleus, whereas others shuttle between the nucleus and cytoplasm (Dreyfuss et al., 1993).

[0058] Antibody staining shows a general nucleoplasmic localization, with little staining in nucleoli and electron-microscopy analysis localized hnRNPs, mainly to perichromatin fibrils. There is no evidence for free (not RNA-bound) hnRNPs in the nucleus. Many hnRNPs show preferential binding to certain RNA sequences, like stretches of identical bases or intron-splice sites. Almost all, including hnRNP-H have a common domain

2. Danen-Van Oorschot, A.A.A.M., Fischer, D., Grimbergen, J.M., Klein, B., Zhuang, S.-M., Falkenburg, J.H.F., Backendorf, C., Quax, P.H.A., Van der Eb, J.A., and Noteborn, M.H.M. (1997). Proceedings National Academy Sciences, USA: 94, 5843-5847.

3. Danen-Van Oorschot, A.A.A.M, Den Hollander, A., Takayama, S., Reed, J., Van der Eb, A.J. and Noteborn, M.H.M. (1997a). BAG-1 inhibits p53-induced but not apoptin-induced apoptosis. Apoptosis 2, 395-402.

4. Dreyfuss, G., Matunis, M.J., Pinol-Roma, S., and Burd, C.G. (1993). HnRNP proteins and the biogenesis of mRNA. Annual Review Biochemistry 62, 289-321.

5. Duke, R.C., Ocjius, D.M., Young, J, D-E. (1996). Cell suicide in health and disease. Scientific American December 1996, 48-55.

6. Durfee, T., Becherer, K., Chen, P.-L., Yeh,S.-H., Yang, Y., Kilburn, A.E., Lee, W.-H., and Elledge, S. J. (1993). The retinoblastoma protein associates with the protein phosphate type I catalytic subunit. Genes and Development 7, 555-569.

7. Earnshaw, W.C., 1995. Nuclear changes in apoptosis. Current Opinion in Cell Biology 7, 337-343.

8. Fields, S. and Song, O.K. (1989). A novel genetic system to detect protein-protein interactions. Nature 340, 245-246.

9. Gamberi, C., Izaurralde, E., Beisel, C., and Mattaj, I.W. (1997). Interaction between the human nuclear cap-binding protein complex and hnRNP F.

10. Hockenberry, D.M. (1994). Bcl-2 in cancer, development and apoptosis. Journal of Cell Science, Supplement 18, 51-55.

11. Hoffman, C.S. and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coili. Gene 57, 267-272.

12. Holzmann, K., Korosec, T., Gerner, C., Grimm, R., Sauermann, G. (1997). European Journal Biochemistry 244, 479-486.

13. Honore, B., Rasmussen, H.H., Vorum, H., Dejgaard, K., Liu, X., Gromov, P., Madsen, P., Gesser, B., Tommerup, N., and Celis, J.E. (1995). Journal of Biological Chemistry 270, 28780-28789.

14. Kerr, J.F.R., Winterford, C.M., and Harmon, B. V. (1994). Apoptosis: Its significance in cancer and cancer therapy. Cancer 73, 2013-2026.

15. Klebe, R.J., Harriss, J.V., Sharp, Z.D., and Douglas, M.G. (1983), A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. Gene 25, 333-341.

16. Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. Cell 88, 323-331.

17. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. CSHL Press, New York, USA.

18. Matunis, M.J., Xing, J., Dreyfuss, G. (1994). The HNRNP-F protein: unique primary structure, nucleic acid-binding properties, and subcellular localization. Nucleic Acids Research 22, 1059-1067.

19. McDonell T.J., Meyn, R.E., Robertson, L.E. (1995). Implications of apoptotic cell death regulation in cancer therapy. Seminars in Cancer Biology 6, 53-60.

20. Noteborn, M.H.M. (1996). PCT application WO 96/41191. Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells as essential characteristic for the development of an anti-tumor therapy.

21. Noteborn, M.H.M., and De Boer, G.F. (1996). Patent USA/no. 030, 335.

22. Noteborn, M.H.M., De Boer, G.F., Van Roozelaar, D., Karreman, C., Kranenburg, O., Vos, J., Jeurissen, S., Zantema, A., Hoeben, R., Koch, G., Van Ormondt, H., and Van der Eb, A.J. (1991). Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. Journal of Virology 65, 3131-3139.

23. Noteborn, M.H.M., Hoeben, R.C., and Pietersen, A. (1997). A gene delivery vehicle expressing the apoptosis-inducing proteins VP2 and/or apoptin. European Patent Application no. 97201121.7

24. Noteborn, M.H.M., Todd, D., Verschueren, C.A. J., De Gauw, H.W.F.M., Curran, W.L., Veldkamp, S., Douglas, A.J., McNulty, M.S., Van der Eb, A.J., and Koch, G. (1994). A single chicken anemia virus protein induces apoptosis. Journal of Virology 68, 346-351.

25. Noteborn, M.H.M., and Zhang, Y. (1997). Methods and means for determining the transforming capability of agents, for determining the predisposition of cells to become transformed and prophylactic treatment of cancer using apoptin-like activity. European Patent Application no. 97439

26. Paulovich, A.G., Toczyski, D., Hartwell, H. (1997). When checkpoints fail. Cell 88, 315-321.

27. Rose, M.D., Winston, F., and Hieter, P. (1990). Methods in yeast genetics. A laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

28. Sachs, L. and Lotem, J. (1993). Control of programmed cell death in normal and leukemia cells: New implications for therapy. Blood 82, 15-21.

29. Sanger, F., Nicklen, S., and Coulsen, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proceedings National Academic Sciences USA 74, 5463-5467.

30. Steller, H. (1995). Mechanisms and genes of cellular suicide. Science 267, 1445-1449.

31. Telford, W.G., King, L.E., Fraker, P.J. (1992). Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. Cytometry 13, 137-143.

CTGAAAATGACATTGCTAATTTCTTCTCACCACTAAATCCAATACGAGTTCATATTGATATTGGA
GCTGATGGCAGAGCCACAGGAGAANCNCCNTGTAGAGTTTGTGACACATGAAGATGCAGTAGCTG
CCATGTCTAAAGATAAAAATAACATGCAACATCGATATATTGAACTCTTCTTGAATTCTACTCCT
GGAGGCGGCTCTGGCATGGGAGGTTCTGGAATGGGAGGCTACGGAAGAGATGGAATGGATAATCA
GGGAGGCTATGGATCAGTTGGAAGAATGGGAATGGGGAACAATTACAGTGGAGGATATGGTACTC
CTGATGGTTTGGGTGGTTATGGCCGTGGTGGTGGAGGCAGTGGAGGTTACTATGGGCANNGCGGC
ATGAGTGGAGGTGGATGGCGTGGGATGTACTGAAAGCAAAAACACCAACATACAAGTCTTGACAA
CAGCATCTGGTCTACTAGACTTTCTTACAGATTTAATTTCTTTTGTATTTTAAGAACTTTATAAT
GACTGAAGGAATGTGTTTTCAANATATTATTTGNGAAAGCAACAGATTGTGATGGGAAAATGTTT
TCNGTTAGTTTATTTGTTGCATACCTTGACTTAAAAATAAATTTTATATTCAAACCNNNAAATTG

Figure 1

C24/hnRNP H homology

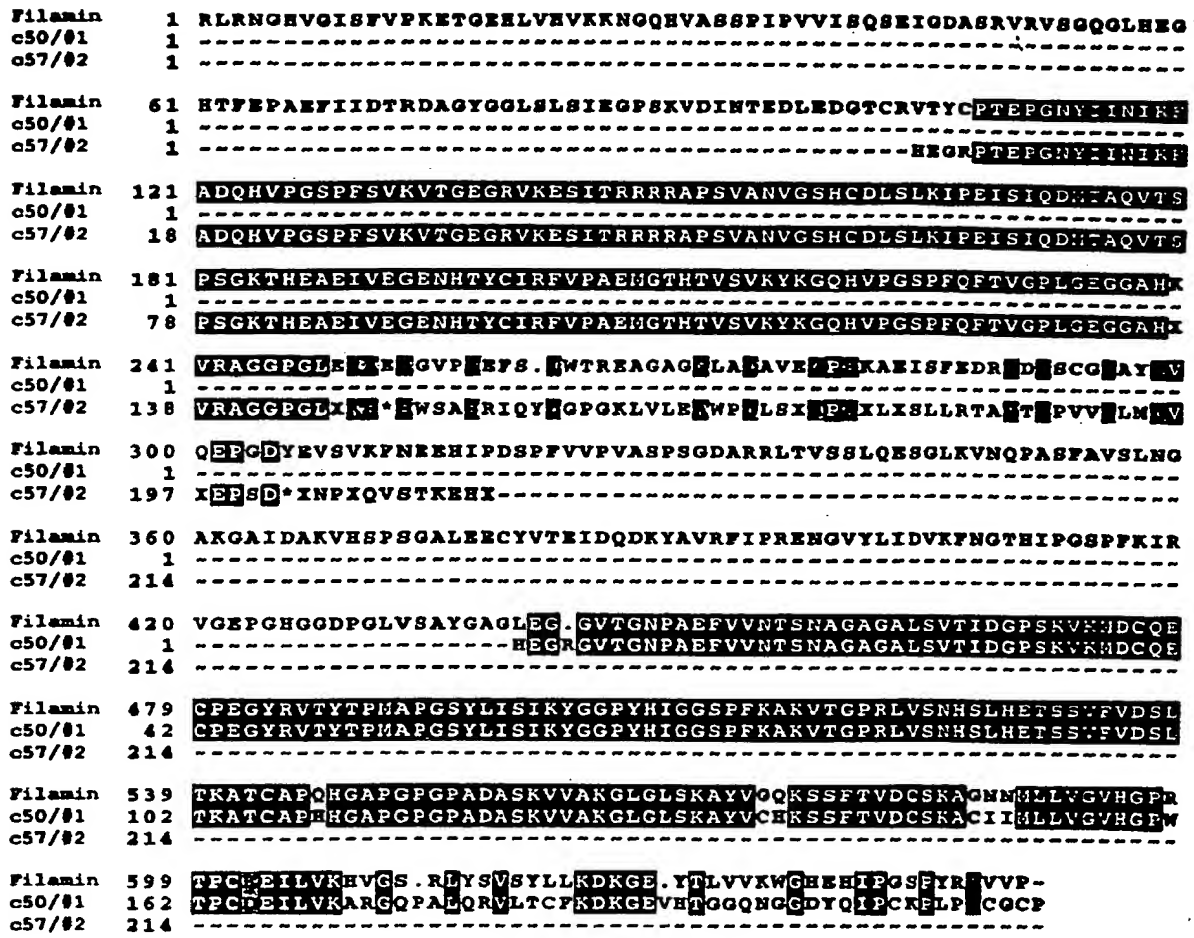

Figure 3